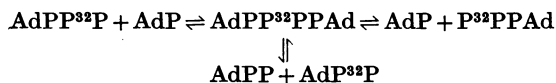


between that of P^{β} and P^{γ} of ATP. However, if the myokinase reaction has an intermediate complex of the type AdPP-PPAd it is possible that P^{β} and P^{γ} of ATP exchange directly without free labelled AdPP being an intermediate according to the scheme



The importance of this reaction could be assessed by a study of the radioactive uptake into P^{β} of ATP and the terminal P of ADP from the P^{γ} of ATP using purified myokinase.

Applicability of the method to other systems. It is clear from the derivation of the equations that this treatment may also be applied to any similar three-compartment system in equilibrium, where one compound participates simultaneously at different rates in two reactions. Sheppard (1948) has made an analysis of the three-compartment system in which, to find two rates in one experiment, the use of two radioactive varieties of the exchanging material is required. A system in which reaction rates in equilibrium conditions could be investigated in this way is the equilibrium of citric, isocitric and aconitic acids in the presence of aconitase.

SUMMARY

1. The incorporation of ^{32}P -labelled orthophosphate into ATP in a steady state during the oxidation of succinate to fumarate by washed suspensions of rat liver mitochondria has been studied. The P^{γ} became radioactive faster than the P^{β} of ATP and the total uptake of ^{32}P could be described by the sum of two exponential terms.

2. A method for the calculation of the rate of the reaction $\text{ADP} + \text{P} \rightarrow \text{ATP}$ and the phosphorylation quotient based on the time course of the uptake of total radioactive P is described. The amount of ^{32}P

incorporated into the P^{β} and P^{γ} as calculated by this method agrees well with the quantities determined experimentally.

3. The method has been applied to the results of Krebs *et al.* (1953) for the oxidation of succinate and citrate and to those obtained in the present work for succinate and pyruvate oxidation. Within an estimated error of 5–10%, the phosphorylation quotient in the conversion of succinate into fumarate was 2.4; for the complete oxidation of pyruvate it was 2.7.

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The Determination of Iron in Plasma or Serum

By T. H. BOTHWELL* AND BARBARA MALLETT

Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford

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Iron circulates in blood plasma as a complex with β_1 -globulin (Schade & Caroline, 1946) which is neither dialysable nor ultrafiltrable (Barkan, 1927). Most methods for estimating this iron involve three stages, (i) incubation of the plasma or serum with dilute hydrochloric acid for periods of 15 min. to several hours to separate the iron from its protein,

(ii) precipitation of plasma proteins with trichloroacetic acid, (iii) formation of a coloured complex with reagents such as *o*-phenanthroline or 2:2'-dipyridyl (Hemmeler, 1951). Recently, Ramsay (1953) has described a method in which the plasma proteins are coagulated in the presence of a buffered solution containing a reducing agent and 2:2'-dipyridyl. In this way a coloured complex is formed under conditions which render reversibility of the

* Nuffield Travelling Fellow in Medicine.

reaction unlikely. Ramsay's results, however, are 30–60 $\mu\text{g.}/100\text{ ml.}$ higher than those previously published, and he has suggested that the lower values found by other workers may be explained by a subsequent chemical or physical reaction between a proportion of the dissolved iron and the precipitated proteins.

In view of the discrepancies between Ramsay's findings and those of others, it seemed important to find out whether all the iron is liberated from its protein by hydrochloric acid, and also whether any of it is subsequently lost by entrainment with the protein precipitated by trichloroacetic acid. These two points have been investigated using radio-active iron. The results of these experiments made it possible to design a method for plasma-iron estimation which incorporates the basic features of other methods but which is more simple and rapid in use.

EXPERIMENTAL

Determination of iron in blood plasma

Glassware. All-glass syringes with stainless-steel needles were used for taking blood. The syringes and other glassware were thoroughly washed and then immersed in chromic acid for several hours. They were then rinsed with hot and cold tap water, distilled water and finally with glass-distilled water.

Reagents. The following reagents were used (all were AR grade and were made up in glass-distilled water): 2N-HCl, 20% (w/v) trichloroacetic acid (TCA), thioglycollic acid, 0.4% (w/v) 2:2'-dipyridyl (0.4 g. dissolved in 5 ml. glacial acetic acid and made up to 100 ml. with glass-distilled water), saturated soln. of sodium acetate, standard soln. of iron (100 mg. of pure iron wire dissolved in dilute H_2SO_4 and oxidized with KMnO_4 ; the volume made up to 100 ml. with glass-distilled water and diluted 1 in 100 to give a soln. for use containing 10 $\mu\text{g.}/\text{ml.}$).

Procedure. 2 ml. 2N-HCl are added to 4 ml. plasma or serum and the mixture is stirred with a glass rod. TCA (2 ml.) is then added and the final mixture stirred vigorously for at least 45 sec. After centrifuging for 20 min. at 2500 rev./min. the supernatant fluid is decanted. The supernatant fluid (5 ml.) is then added to a tube containing 2 drops of thioglycollic acid, 0.5 ml. 0.4% 2:2'-dipyridyl and 2.5 ml. sat. soln. of sodium acetate and the mixture shaken thoroughly. The colour intensity is measured in a Hilger New Biochem. absorptiometer with a 520 $\text{m}\mu$. filter. This is calibrated by means of solutions of iron containing from 2 to 10 $\mu\text{g. Fe}$ together with 1.25 ml. of 2N-HCl and 1.25 ml. 20% TCA; the volume is made up to 5 ml. and the mixture treated as above. A reagent blank is also measured.

Comment on procedure

2:2'-Dipyridyl, first used in the estimation of iron in biological material by Hill (1930), forms a stable coloured complex with iron within the pH range 3–9 (Moss & Mellon, 1942). In the method described above, thioglycollic acid, which rapidly reduces iron from ferric to the ferrous state at pH between 4.5 and 4.9 (Laurell, 1952), was used as the reducing agent and the solution buffered with a saturated

soln. of sodium acetate. Experiments were carried out to find out the importance of the various factors in the colour reaction and the accuracy of the method.

Accuracy of the method (Table 1). The error in measurement was relatively greater with low concentrations of iron but was small enough to allow for moderate accuracy over a wide range of iron concentrations.

Clarity of the centrifuged solution. Hemmeler (1951) has reported difficulty in obtaining a clear supernatant after centrifuging. However, in the present method it was found that vigorous stirring of the mixture before centrifuging yields a clear supernatant that gave readings in the colorimeter no higher than a blank made up with the same reagents and glass-distilled water.

Importance of pH. The mean pH of the final soln. was 4.7 (range 4.54–4.77) in forty-eight estimations carried out with a glass electrode. By varying the experimental conditions it was also shown that the final pH was not critical enough to necessitate the titration of each sample as is described in some methods (Table 2).

Efficiency of thioglycollic acid as a reducing agent. The present method was compared with procedures in which the colour was developed by different techniques. The supernatant fluid was treated in one of two ways. In the first, 0.1 ml. 0.1M- Na_2SO_3 was used as a reducing agent and the

Table 1. *Accuracy of 2:2'-dipyridyl in determining iron in standard solutions*

Ten estimations for each concentration.

Fe in standard solutions ($\mu\text{g.}/100\text{ ml.}$)	Coefficient of variation
20	7.0
80	2.4
160	0.9

Table 2. *Effect of variations in pH on the recovery of iron from standard solutions*

Solutions contained 20–400 $\mu\text{g. Fe}/100\text{ ml.}$ In this and subsequent tables results are given as mean \pm s.d.

Range of pH	No. of estimations	Fe recovered (%)
3.7–4.5	18	98.9 \pm 2.07
4.5–5.2	20	100.6 \pm 1.50

Table 3. *Efficiency of thioglycollic acid as a reducing agent*

Each estimation was carried out in duplicate on two samples, of which one was treated by the present method and the other either with Na_2SO_3 or with HNO_3 and KCNS (for details see text). The iron content of the samples varied from 30 to 224 $\mu\text{g.}/100\text{ ml.}$ (mean 106 $\mu\text{g.}/100\text{ ml.}$).

Alternative treatment of iron in supernatant	No. of duplicate estimations	Mean difference between each pair of estimations ($\mu\text{g. Fe}/100\text{ ml.}$)
Reduced with 0.1M- Na_2SO_3	10	3.4 \pm 2.4
Oxidized by boiling with HNO_3 and converted into thiocyanate	10	5.6 \pm 3.2

solution buffered to pH 4.7 with saturated soln. of sodium acetate. In the second, the iron was oxidized by boiling for 30 min. with 0.1 ml. HNO_3 . By the addition of KCNS the iron was converted into thiocyanate and extracted with amyl alcohol. In both sets of experiments there was good agreement with results obtained by the present method (Table 3).

Time taken for development of final colour. In thirty samples and the reagent blank the intensity of colour was read immediately after preparation, then the samples were covered with cellophan, incubated at 37° for 1 hr., and the reading repeated. The mean difference in plasma iron concentration after re-reading was only $0.9 \mu\text{g./100 ml.}$ (s.d. $\pm 1.2 \mu\text{g./100 ml.}$).

Reproducibility and effect of anticoagulants. The method gave reproducible results unaffected by the use of anti-coagulants (Table 4).

Recovery of added iron. It was shown that iron added to plasma either as a salt or bound to β_1 -globulin (supplied by Cutter Laboratories, U.S.A.) could be recovered quantitatively (Table 5).

Effect of haemolysis. Red cells of known haemoglobin content were washed 3 times with 0.9% NaCl and were then haemolysed with water. Their approximate iron content was calculated on the assumption that iron forms 0.34% of the haemoglobin molecule by weight (Bernhart & Skeggs, 1943). Small volumes of haemolysed solution with iron content varying from approximately 60 to $600 \mu\text{g./100 ml.}$ were then added to twenty plasma samples and the mixtures were analysed for iron content. The mean difference in plasma iron concentration after the addition of haemolysed erythrocytes was only $3.5 \mu\text{g./100 ml.}$ (s.d. $\pm 4.5 \mu\text{g./100 ml.}$).

Comparison with other methods. There was good agreement with the results obtained by two other methods for plasma iron determination, namely that of Ramsay (1954) and that of Barkan & Walker (1940), which is similar in principle to

Table 4. *Reproducibility of results obtained on heparinized and oxalated plasma and serum*

The iron concentration of the samples varied from 18 to $234 \mu\text{g./100 ml.}$ (mean $106 \mu\text{g./100 ml.}$).

	No. of estimations	Mean difference between each pair of estimations ($\mu\text{g. Fe/100 ml.}$)
Duplicate samples of heparinized plasma	30	2.6 ± 3.8
Comparison between heparinized plasma and oxalated plasma	15	3.2 ± 3.9
Comparison between heparinized plasma and serum	15	2.0 ± 1.9

Table 5. *Recovery of iron added to plasma samples*

Form in which iron was added to 3 ml. plasma samples	Quantity of added iron ($\mu\text{g./100 ml.}$)	No. of estimations	Recovery (%)
Standard ferric sulphate solutions	2-6	25	98.6 ± 4.2
Ferric sulphate solution buffered to pH 6.4 with sodium citrate and bound to β_1 -globulin	2	12	99.0 ± 4.2

the present method but which has longer time intervals and involves the use of hydrazine sulphate as reducing agent and o-phenanthroline for the development of the colour (Table 6).

Experiments with ^{59}Fe

Experiments were carried out to determine whether all the iron was detached from the metal-binding protein by precipitating the proteins with acids.

Administration of ^{59}Fe . $^{59}\text{FeCl}_3$ of high specific activity ($1 \mu\text{C/0.5 } \mu\text{g. Fe}$), obtained from an American source through A.E.R.E., Harwell, was given in tracer doses of 10-20 μC to a number of subjects in three ways:

(a) Injected intravenously after having been added *in vitro* to a solution of β_1 -globulin as described by Huff *et al.* (1951).

(b) Injected intravenously after preliminary incubation with the patient's plasma as described by Huff *et al.* (1950).

(c) Fed by mouth together with approximately 5 mg. of unlabelled ferrous ascorbate.

By taking blood samples at periods of 10-60 min. after the administration of ^{59}Fe it was possible to obtain plasma labelled with ^{59}Fe .

Separation of iron from protein. The radioactivity in plasma was compared with that present in the supernatant fluid from samples which had been treated in various ways with HCl and TCA (Table 7). The counting was done in a scintillation counter (type 1186 on loan from A.E.R.E., Harwell) by the technique of Badenoch & Callender (1954). With the quantities of ^{59}Fe used in these studies the maximum counting error was $\pm 5\%$.

RESULTS AND DISCUSSION

Table 7 shows that complete recoveries were obtained in 101 estimations. There was no apparent difference between the results obtained after allowing the plasma to stand for varying periods with HCl and trichloroacetic acid and those after

Table 6. *Comparison of results obtained by present method with those of two published methods*

The iron concentration of the plasma samples varied between 30 and $224 \mu\text{g./100 ml.}$ (mean $103 \mu\text{g./100 ml.}$). Ten duplicate estimations were carried out for each comparison.

Method	Mean difference between each pair of estimations ($\mu\text{g. Fe/100 ml.}$)
Present method compared with Barkan & Walker's (1940) method	3.0 ± 2.6
Present method compared with Ramsay's (1954) modified technique	4.6 ± 2.0

Table 7. Recoveries of ^{59}Fe obtained from plasma treated with 2N-HCl and 20% trichloroacetic acid

(A) Samples were mixed with HCl and allowed to stand for 10–60 min. Trichloroacetic acid was then added and the samples were mixed thoroughly and allowed to stand for a further 5–60 min. (B) Samples were treated in the same way as in (A) except that they were not left to stand after the addition of HCl and trichloroacetic acid.

Method of administration of ^{59}Fe	Treatment of plasma samples	No. of estimations	% recoveries of radioiron from supernatant fluid after centrifuging
^{59}Fe bound to β_1 -globulin and injected intravenously (six subjects)	A	28	101.2 \pm 3.7
	B	16	100.8 \pm 5.2
^{59}Fe incubated with plasma and injected intravenously (three subjects)	A	16	97.8 \pm 6.2
	B	15	101.0 \pm 7.1
^{59}Fe fed by mouth (three subjects)	A	7	107.1 \pm 7.6
	B	19	104.7 \pm 6.6

immediate mixing, nor did the initial manner of binding the iron to the β_1 -globulin affect the results. However, in four estimations in which the plasma proteins were precipitated with trichloroacetic acid without having been treated with HCl, recoveries of only about 65% were obtained.

By giving tracer doses of ^{59}Fe of high specific activity parenterally or orally, it was possible to obtain plasma samples in which the transport iron attached to β_1 -globulin was labelled with radioactivity. It was shown that this iron was rapidly and completely separated from its protein attachment after thorough mixing with dilute HCl and trichloroacetic acid. As there is no reason to believe that radioactive iron behaves differently from ordinary iron it seems valid to assume that the many methods which use such a technique for the separation of iron from protein should give reliable results.

The present technique for iron estimation in plasma or serum, although it involves no new principles, has the advantage of simplicity and speed. The results of duplicate analyses have shown good agreement, and the occurrence of haemolysis has not affected the results significantly. A comparison of the results obtained by this method with those obtained by Barkan & Walker (1940) showed satisfactory agreement. In addition, it was also found that the present method gave results no lower than those obtained with Ramsay's modified technique (1954).

SUMMARY

1. A simple and rapid modification of previous techniques for the estimation of iron in plasma is described.

2. Recovery experiments with ^{59}Fe showed that iron is effectively separated from β_1 -globulin in plasma by treatment with dilute hydrochloric acid and trichloroacetic acid.

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